Identification of Amino Acids in the N-Terminal SH2 Domain of Phospholipase Cγ1 Important in the Interaction with Epidermal Growth Factor Receptor†

James R. Gergel,‡ Dennis J. McNamara,§ Ellen M. Dobrusin,§ Guochang Zhu,|| Alan R. Saltiel,|| and W. Todd Miller*,‡

Department of Physiology and Biophysics, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794, and Departments of Medicinal Chemistry and Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105

Received July 14, 1994; Revised Manuscript Received September 21, 1994⊗

ABSTRACT: Photoaffinity labeling and site-directed mutagenesis have been used to identify amino acid residues of the phospholipase Cγ1 (PLCγ1) N-terminal SH2 domain involved in recognition of the activated epidermal growth factor receptor (EGFR). The photoactive amino acid *p*-benzoylphenylalanine (Bpa) was incorporated into phosphotyrosine-containing peptides derived from EGFR autophosphorylation sites Tyr992 and Tyr1068. Irradiation of these labels in the presence of SH2 domains showed cross-linking which was time-dependent and specific; labeling was inhibited with non-Bpa-containing peptides from EGFR in molar excess. The phosphotyrosine residue on the peptides was important for SH2 recognition, as dephosphorylated peptides did not cross-link. Radiolabeled peptides were used to identify sites of cross-linking to the N-terminal SH2 of PLCγ1. Bpa peptide−SH2 complexes were digested with trypsin, and radioactive fragments were purified by HPLC and analyzed by Edman sequencing. These experiments showed Arg562 and an additional site in the $\alpha_A-\beta_B$ region of the SH2 domain, most likely Glu587, to be labeled by the Tyr992-derived peptide. Similar analysis of the reaction with the Tyr1068-derived photoaffinity label identified Leu653 as the cross-linked site. Mutation of the neighboring residues of Glu587 decreased photo-cross-linking, emphasizing the importance of this region of the molecule for recognition. These results are consistent with evidence from the v-Src crystal structure and implicate the loop spanning residues Gln640−Ser654 of PLCγ1 in specific recognition of phosphopeptides.

Epidermal growth factor (EGF) binds to a specific transmembrane receptor (EGFR, M_r = 170 000), functioning as a signal which, when transduced inside the cell, results in cell proliferation or differentiation [reviewed in Fantl *et al.* (1993)]. Binding of EGF to the extracellular domain of EGFR induces receptor oligomerization (Ullrich & Schlessinger, 1990). The cytoplasmic kinase domain subsequently autophosphorylates five tyrosine residues toward the carboxyl terminus of the receptor (tyrosines 992, 1068, 1086, 1148, and 1173) in an intermolecular process (Fantl *et al.*, 1993; Ullrich & Schlessinger, 1990). These phosphotyrosine residues allow several proteins to interact with the EGFR through conserved phosphotyrosine recognition sequences known as Src homology 2 (SH2) domains (Anderson *et al.*, 1990; Margolis *et al.*, 1990). These domains, originally identified in the product of the *v-src* oncogene, are present in a variety of proteins involved in signal transduction (Pawson *et al.*, 1993; Mayer & Baltimore, 1993). Presumably, extracellular signals are transduced by SH2-containing proteins binding to specific phosphotyrosine residues on proteins upstream in the pathway. Signal transduction could proceed through three mechanisms, or any combination of them: an altered cellular location of the SH2 protein, an induced conformational change, or phosphorylation leading to the activation of the SH2 protein (Pawson *et al.*, 1993; Mayer & Baltimore, 1993).

Phospholipase Cγ1 (PLCγ1) is a member of the EGF signal transduction pathway whose activity is regulated by tyrosine phosphorylation (Rhee & Choi, 1993). PLCγ1 has two SH2 domains which allow it interact with the EGFR, both near the middle of the PLCγ1 primary sequence between the conserved X and Y boxes (Rhee & Choi, 1993). They will be denoted the N- and C-terminal SH2 domains (N-SH2 and C-SH2) on the basis of their location relative to each other. After binding to the phosphotyrosine residues on the receptor, PLCγ1 is itself phosphorylated by the EGFR. This modification appears to be essential to achieve complete activation of PLCγ1 for hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce the signaling molecules diacylglycerol and inositol 1,4,5-trisphosphate (Rhee & Choi, 1993).

The phosphotyrosine residues are the main determinants of binding specificity (Anderson *et al.*, 1990; Margolis *et al.*, 1990). Further, it is the phosphotyrosines at sites 992 and 1068 which are primarily involved in the association of PLC to the EGFR. *In vitro* experiments using PLCγ1 SH2 fusion proteins have shown the sequence surrounding Tyr992 to be the highest affinity binding site on the EGFR, followed by Tyr1068 (Rotin *et al.*, 1992). Moreover, binding of PLCγ1 SH2 domains to purified EGFR can be reduced by competition with low concentrations of short phosphorylated

† This work was supported by a grant from the National Institutes of Health (CA5853001) to W.T.M.

* To whom correspondence should be addressed at the Department of Physiology and Biophysics, Basic Science Tower T-6, School of Medicine, SUNY at Stony Brook, Stony Brook, NY 11794-8661 (telephone 516-444-3533; Fax 516-444-3432).

‡ State University of New York at Stony Brook.

§ Department of Medicinal Chemistry, Warner-Lambert Co.

|| Department of Signal Transduction, Warner-Lambert Co.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1994.

0006-2960/94/0433-14671$04.50/0

peptides which mimic EGFR autophosphorylation sites Tyr992 and Tyr1068 (McNamara *et al.*, 1993). Even relatively high concentrations of nonphosphorylated peptides showed no effect on PLCγ1 SH2−EGFR interaction (McNamara *et al.*, 1993). Recognition of phosphotyrosine is clearly important, but it is presently unknown which amino acid residues in the SH2 domains of PLCγ1 are involved in EGFR recognition and binding. The identity of the residues involved in receptor binding is important for the design of inhibitors of SH2-mediated associations, for instance (Mayer & Baltimore, 1993).

Details concerning protein structure and function, particularly involving the active sites of enzymes or binding sites such as SH2 domains, can be revealed by photoinduced affinity labeling [reviewed in Brunner (1993)]. Proteolytic digestion of the cross-linked protein permits localization and sequencing of the binding site(s). The modified amino acid *p*-benzoylphenylalanine (Bpa), when substituted into a synthetic peptide which binds to a protein site, will cross-link nonspecifically with nearby residues upon photoactivation (Kauer *et al.*, 1986; Miller & Kaiser, 1988; Dormán & Prestwich,1993). Because of the high reactivity of the photolytically generated intermediate, it will label even normally unreactive amino acids (Miller, 1991). Accordingly, Bpa-containing peptides mimicking two EGFR autophosphorylation sites were used to label the SH2 residues involved in phosphotyrosine binding.

MATERIALS AND METHODS

Materials. N^{α}-9-Fluorenylmethoxycarbonyl- (Fmoc-) protected amino acids, including N^{α}-Fmoc-4-benzoyl-L-phenylalanine, were purchased from Bachem Bioscience and Applied Biosystems. Other reagents for peptide synthesis were from Aldrich or Burdick and Jackson. Thrombin, potato acid phosphatase, and TPCK-treated trypsin were purchased from Sigma. ScintiVerse II scintillation fluid was obtained from Fisher. Centricon and Microcon concentrators were from Amicon. Expression of either or both of the phospholipase Cγ1 SH2 domains in *Escherichia coli* as fusion proteins with glutathione *S*-transferase (GST) and purification on glutathione−agarose (Molecular Probes, Inc.) were carried out as described previously (Smith & Johnson, 1988; Zhu *et al.*, 1992). Removal of the GST portions of the fusion proteins (while bound to GST−agarose) was carried out by incubation with 1:200 (w/w) thrombin (30 min, 25 °C).

Peptide Synthesis. The peptides were prepared by standard solid-phase methodology on an ABI 431A peptide synthesizer using an Fmoc protection strategy (Stewart & Young, 1984). Phosphotyrosine-containing peptides 992, 1068, 992N-Bpa, 992C-Bpa, and 1068C-Bpa were synthesized by an on-the-resin phosphorylation procedure previously described (McNamara *et al.*, 1993) with the following modifications for the Bpa-containing peptides. The terminal amino acid was incorporated as its Fmoc-protected derivative. Phosphorylation of this resin-bound, protected peptide was followed by removal of the N-terminal Fmoc group [20% piperidine/*N*-methylpyrrolidinone (NMP)] and then treatment with acetic anhydride (5 equiv) in NMP. The peptides were cleaved from the resin and the protecting groups removed by treatment with a solution of trifluoroacetic acid (TFA), thioanisole, and water (9.5 mL, 0.25 mL, 0.25 mL). The peptides were purified by preparative C18 reverse-phase HPLC. Peptides 992N-Bpa, 992C-Bpa, and 1068C-Bpa were greater than 95% pure by analytical HPLC and displayed amino acid analyses (*p*-benzoylphenylalanine not observed), proton NMR spectra, and fast atom bombardment mass spectra (FAB-MS) or electrospray MS supportive of their structures. FAB-MS of peptide 992N-Bpa, $(M + H)^+$ found 1493.4, calculated for $C_{67}H_{90}N_{13}O_{24}P + H$, 1492.6; electrospray mass spectrum of peptide 992C-Bpa, $(M + H)^+$ found 1509.2, calculated for $C_{66}H_{86}N_{13}O_{26}P + H$, 1509.5; FAB-MS of peptide 1068C-Bpa, $(M + H)^+$ found 1666.9, calculated for $C_{79}H_{105}N_{14}O_{24}P + H$, 1665.7.

The radiolabeled peptides were prepared as above but were acetylated with [^{3}H]acetic anhydride (5 equiv, 500 mCi/mmol; Amersham) (Miller, 1991). The radiolabeled peptides were purified on an analytical C18 reverse-phase HPLC column and mixed with pure nonradiolabeled peptide to the desired specific activities. For peptide 992N-Bpa, 3.77 mg (specific activity of 23.2 μCi/mg) of material was obtained (chemical purity 96% by HPLC) which was dissolved in 0.38 mL of H_2O for the cross-linking studies. For peptide 1068C-Bpa, 1.57 mg (specific activity of 20.1 μCi/mg) of material was obtained (chemical purity 95% by HPLC) which was dissolved in 0.20 mL of 50% DMSO/H_2O for the cross-linking studies.

Photoaffinity Labeling. Irradiations were carried out at 4 °C in a Rayonet RMR-500 photochemical reactor fitted with eight RMR 3500-Å lamps (Southern New England Ultraviolet Co., Hamden, CT), as described previously (Miller, 1991). The samples were clamped 2 cm from the lamps in borosilicate glass tubes. To measure half-times of photolysis, the photoactive peptides were dissolved in 50 mM Tris, pH 7.5, and were photolyzed for varying amounts of time. Aliquots were removed and absorbances at 262 nm were measured (Miller & Kaiser, 1988). Photo-cross-linking to the purified SH2 fusion proteins was carried out under similar conditions. Aliquots were removed after various times of photolysis, mixed with SDS−sample buffer, heated at 100 °C, and applied to a 15% SDS−polyacrylamide gel. Dephosphorylated peptide 992N-Bpa was prepared by incubation of 102 μmol of peptide with 2.1 units of potato acid phosphatase in 50 mM sodium citrate, pH 5.0, for 90 min. The reaction was followed by analytical C18 HPLC on a Vydac C18 column, and dephosphorylated peptide 992N-Bpa was purified using the following mobile phases for gradient elution: (A) 0.1% (v/v) trifluoroacetic acid (TFA) and (B) acetonitrile/water, 90:10 (v/v), containing 0.1% TFA. Peptides were eluted with a linear gradient of 5−40% solvent B in 40 min, at a flow rate of 1.0 mL/min; dephosphorylated peptide 992N-Bpa eluted ≈0.5 min after the phosphorylated peptide.

Identification of Modified Sites. Two preparative-scale photo-cross-linking reactions were carried out. In the first, N-SH2 domain (38.4 nmol) was incubated with 1.7 μmol of peptide 992N-Bpa (7.6×10^4 dpm/nmol) in 50 mM Tris (pH 7.5). In the other reaction, N-SH2 (17 nmol) was incubated with 625 nmol of peptide 1068C-Bpa (1.2×10^5 dpm/nmol) in 50 mM Tris (pH 7.5). In each case, the solution was irradiated at 350 nm for 20 min at 4 °C. To remove excess photoaffinity label, the reaction mixture was transferred to Microcon-3 concentration units (Amicon; molecular weight cutoff, 3000). After being washed, the cross-linked SH2 domain was dissolved in 100 mM NH_4HCO_3 and incubated with 1:100 (w/w) trypsin. Cleavage was carried out at 37 °C for 20 h; the progress of a trypsin

digestion of unmodified SH2 domain indicated that overnight cleavage was necessary for digestion (see Figure 2A).

Trypsin was removed by filtration through a Centricon-10 ultrafiltration unit (molecular weight cutoff, 10 000). The unit was washed until ≈98% of the radioactivity applied to the Centricon-10 had passed through to the filtrate. HPLC analysis was carried out on an ISCO 2350 HPLC system. The filtrates from the larger scale photoaffinity labeling reactions were divided into two portions and analyzed in two injections on a Vydac C18 analytical column with a mobile phase composed of 0.1% trifluoroacetic acid (TFA) in acetonitrile. Peptides were eluted with a linear gradient of 0−80% acetonitrile in 90 min, at a flow rate of 1.0 mL/min.

Radioactive peaks were concentrated *in vacuo* in a Speedvac concentrator (Savant) and analyzed by Edman peptide sequencing using an Applied Biosystems Model 475A pulsed liquid protein sequencer. The samples were collected onto an Ultrafree device containing Immobilon-CD membranes (Millipore) by centrifugation at 800 rpm for 15 min. The samples were washed with 120 μL of 35 mM NH_4OH according to the manufacturer's specifications. Precycling prior to normal sequencing cycles involved the following successive treatments: TFA, dry, *n*-heptane, butyl chloride, dry, trimethylamine (TMA) buffer, dry, phenyl isothiocyanate in *n*-heptane, TMA buffer, dry, *n*-heptane, ethyl acetate. For each peak sequenced (peaks b1, b3, b4, c2, and c3), portions of the filtrate from the Ultrafree device, of the washes, and of the precycling samples were analyzed by scintillation counting. All such samples contained negligible radioactivity (cpm − blank ≤ 10). At each sequencing cycle, one-half of the phenylthiohydantoin-amino acid was identified by HPLC, and the remainder was dissolved in ScintiVerse fluid (Fisher) and analyzed by scintillation counting. Peaks from the corresponding positions in the gradient from both injections gave identical peptide sequences.

Cross-Linking to Mutant SH2 Domains. SH2 domains (3 μM, mutant or wild type) were irradiated for 5 min at 4 °C together with 14 μM ^3H peptide 992N-Bpa (7.6×10^4 dpm/nmol). Reaction mixtures were incubated with glutathione−agarose, and the agarose beads were centrifuged, washed, and resuspended in Tris buffer. After addition of ScintiVerse fluid, the ^3H bound to glutathione−agarose was quantified by scintillation counting. In the absence of GST−SH2 fusion protein, less than 2% of the ^3H peptide 992N-Bpa remained bound to the glutathione−agarose beads after washing. Experiments without added peptide indicated that approximately 88% of the SH2 domain bound to glutathione−agarose under these conditions, as judged by measurements of protein concentration using the Pierce Coomassie microassay.

RESULTS

Photoaffinity Labels. Three peptides containing the photoreactive Bpa amino acid were constructed by standard solid-phase synthetic methodologies [reviewed in Stewart and Young (1984)]. Each peptide was acetylated on the N-terminus by reaction with acetic anhydride. Two were based on the EGFR autophosphorylation site at position 992, one with the Bpa immediately N-terminal to the phosphotyrosine (992N-Bpa) and the other with the label C-terminal (992C-Bpa). The third peptide resembled the autophosphorylation site at position 1068, with an immediately C-terminal label

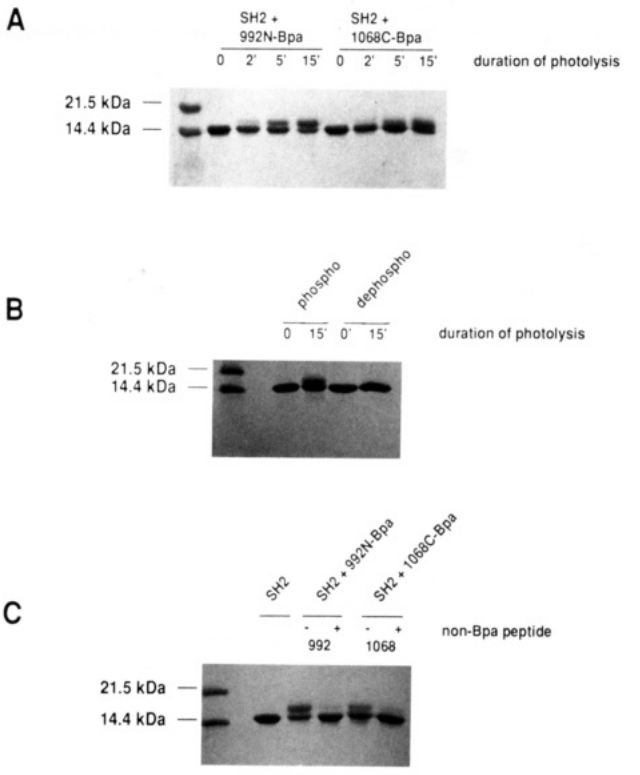

FIGURE 1: Photoaffinity labeling of the PLCγ1 N-terminal SH2 domains. (A) SH2 domain (3.1×10^{-4} M) and peptide C (1.4×10^{-3} M) were incubated in 50 mM Tris, pH 7.5. The solution was photolyzed at 4 °C, and aliquots were removed at 0, 2, 5, and 15 min of photolysis. These samples were analyzed by SDS−PAGE on a 15% gel and detected by Coomassie staining. A separate reaction containing SH2 domain (3.1×10^{-4} M) and peptide D (1.6×10^{-3} M) was photolyzed and analyzed in a similar manner. Lanes: 1, low molecular weight standards; 2−5, peptide C reaction at 0, 2, 5, and 15 min of photolysis; 6−9, peptide D reaction at 0, 2, 5, and 15 min of photolysis. (B) SH2 domain (3.7×10^{-4} M) and peptide C (9×10^{-4} M) were photolyzed as described in (A). Lanes: 1, low molecular weight standards; 2, sample before photolysis; 3, sample after 15 min of photolysis [in a separate reaction SH2 domain was photolyzed with dephosphorylated peptide C (9×10^{-4} M)]; 4, sample before photolysis; 5, sample after 15 min of photolysis. (C) SH2 domain (3.7×10^{-4} M) was irradiated for 15 min in the presence of photoaffinity labels (4×10^{-4} M) and in the presence and absence of non-Bpa peptides (4×10^{-3} M). Lanes 1, SH2 domain alone; 2, SH2 plus peptide 992N; 3, same as lane 2 plus peptide 992; 4, SH2 plus peptide 1068C; 5, same as lane 4 plus peptide 1068.

(1068C-Bpa). The sequences of the peptides are (with the Bpa in boldface)

992C-Bpa: Ac-Asp-Ala-Asp-Glu-Tyr992(PO_3H_2)-**Bpa**-Ile-Pro-Gln-Gln-Gly

992N-Bpa: Ac-Asp-Ala-Asp-**Bpa**-Tyr992(PO_3H_2)-Leu-Ile-Pro-Gln-Gln-Gly

1068C-Bpa: Ac-Phe-Leu-Pro-Val-Pro-Glu-Tyr1068(PO_3H_2)-**Bpa**-Asn-Gln-Ser-Val

Photoreactivity of the purified Bpa-labeled peptides was confirmed by irradiating the peptide in the presence of buffer alone. The loss of absorbance at 262 nm as a function of photolysis indicated a half-time of roughly 5 min (data not shown).

Photo-Cross-Linking to PLCγ1 SH2 Domains. PLCγ1 SH2 domains were expressed in *E. coli* as fusion proteins with glutathione *S*-transferase (GST) (Smith & Johnson,

1988). Prior to use, the GST portion of the proteins was removed by thrombin cleavage. Fragments consisting of either or both PLCγ1 SH2 domains were incubated with a 5-fold molar excess of Bpa-labeled peptides in 50 mM Tris (pH 7.5). These solutions were irradiated at 350 nm for various times at 4 °C. Cross-linking of the photolabels to the SH2 domains, visualized on Coomassie-stained SDS–PAGE gels, proceeded in a time-dependent manner with a half-time of roughly 5 min (see above). Qualitatively, cross-linking was readily apparent in the form of an additional band $\approx$1.5 kDa larger than the unlabeled SH2 domains (Figure 1A). Effectively maximal labeling, approaching 60%, was achieved after 15 min. Reactions between the SH2 domain and the photoactive peptides could not be driven to 100% cross-linking, even with the addition of higher concentrations of peptide or irradiation for longer periods of time (data not shown).

All three peptides cross-linked to both the C- and N-terminal SH2 regions of PLCγ1. However, reactions between either 992N-Bpa or 1068C-Bpa and the N-SH2 region showed the greatest efficiency, and thus these combinations were followed throughout the study (Figure 1A).

Specificity of Photoaffinity Labeling. Potato acid phosphatase was used to dephosphorylate Bpa-containing peptides to test the importance of phosphotyrosine in the interaction. High-performance liquid chromatography confirmed that dephosphorylation was complete (data not shown). HPLC was also used to purify the dephosphorylated form of peptide 992N-Bpa. Photo-cross-linking between the dephosphorylated peptide and the N-terminal SH2 domain was absent (Figure 1B), presumably because the SH2 domain failed to bind peptide 992N-Bpa in the absence of a phosphotyrosine residue.

In order to determine the specificity of photolabeling, the reactions with N-SH2 were carried out in the presence of Bpa-containing peptides and a 10-fold molar excess of non-Bpa-substituted peptides from EGFR, peptides 992 and 1068:

992: Asp-Ala-Asp-Glu-Tyr992(PO_3H_2)-Leu-Ile-Pro-Gln-Gln-Gly

1068: Phe-Leu-Pro-Val-Pro-Glu-Tyr1068(PO_3H_2)-Ile-Asn-Gln-Ser-Val

As seen in Figure 1C, photolabeling was inhibited by the presence of these peptides, indicating that labeling is dependent on the specific binding of the peptide by the SH2 domain. In addition, photo-cross-linking of peptides 992N-Bpa and 1068C-Bpa to the Src SH2 domain was less efficient than to the PLCγ1 SH2 domains, consistent with predictions of specificity for the SH2 domains (Songyang *et al.*, 1993) (Table 2 and see discussion below). Cross-linking of a Bpa-substituted analog of the c-Src C-terminal tail (Glu-Pro-Bpa-phosphoTyr-Gln-Pro-Gly-Glu-Asn-Leu) was also of low efficiency (data not shown).

Sequencing of the Photo-Cross-Linked SH2 Domain. To identify the amino acid residues of the PLCγ1 N-terminal SH2 domain which were modified by the peptide photoaffinity labels, preparative-scale cross-linking reactions were carried out using peptides which had been acetylated with [^{3}H]acetic anhydride. After irradiation, the cross-linked complexes were proteolyzed with trypsin to produce peptide fragments suitable for HPLC analysis. In the first preparative

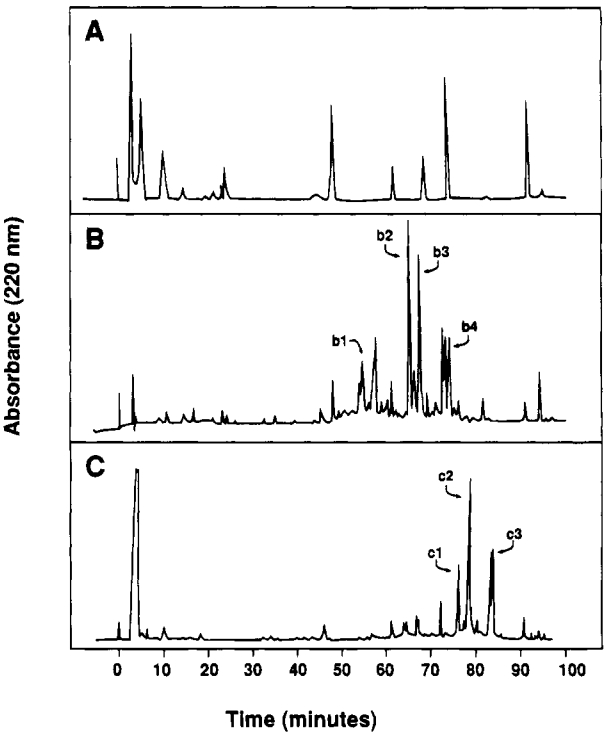

FIGURE 2: HPLC analysis of trypsin-digested SH2 domains. Conditions for the digestions and for HPLC are given in the Materials and Methods section. (A) Trypsin digestion of the N-terminal SH2 domain of phospholipase Cγ1. (B) Digestion of the complex between the SH2 domain and peptide 992N. Positions of radioactive peaks are indicated. (C) Digestion of the complex between the SH2 domain and peptide 1068C. Positions of radioactive peaks are indicated.

reaction, the N-SH2 domain was incubated with ^{3}H-acetylated peptide 992N-Bpa, and the solution was irradiated at 350 nm for 20 min at 4 °C. After being washed, the cross-linked SH2 domain was dissolved in NH_4HCO_3 and incubated with trypsin. Cleavage was carried out at 37 °C for 20 h; the progress of a trypsin digestion of unmodified SH2 domain had indicated that overnight incubation was necessary for digestion (Figure 2A).

Trypsin was removed by filtration in a Centricon-10 unit; ~98% of the radioactivity was recovered in the filtrate, indicating that the radioactivity was associated with small (M_r < 10 000) tryptic fragments. The filtrate was divided into two portions and analyzed by HPLC in two injections on an analytical C18 reverse-phase column. Tryptic peptides were purified using a linear gradient of CH_3CN and an aqueous solution containing trifluoroacetic acid (TFA). Fractions containing peaks of absorbance at 220 nm were collected and analyzed by scintillation counting after 5% of each sample was dissolved in scintillation fluid. Prior to analysis of the preparative reactions, analytical-scale photo-cross-linking with each peptide was carried out (using 1 nmol of SH2 domain) in order to optimize HPLC gradient conditions. The chromatograms of these reactions were similar to those observed for the larger scale reactions (data not shown). Major peaks of radioactivity from the trypsin digest of the modified SH2 domain eluted at 54.6 min (peak b1), 65.1 min (peak b2), 67.3 min (peak b3), and 73.8 min (peak b4) (Figure 2B). Minor radioactive peaks in the HPLC chromatogram may arise because multiple points of attachment into peptide chains are possible with benzophenones (Dormán & Prestwich, 1993), and multiple stereoisomers may be formed. In addition, multiple peaks may be observed

Table 1: Sequenced Peptides from the Cross-Linked PLCγ SH2 Domain[a]

	peak b1		peak b3		peak b4		peak c2		peak c3	
cycle	amino acid (pmol)	radio-activity (cpm)	amino acid (pmol)	radio-activity (cpm)	amino acid (pmol)	radio-activity (cpm)	amino acid (pmol)	radio-activity (cpm)	amino acid (pmol)	radio-activity (cpm)
1	D (184)	14	L (647)	41	D (40)	29	G (10), L (7), F (3)	2810	G (15), D (3), F (4)	40
2	G (98)	20	L (466)	52	A (7)	40	S (42)	184	L (30)	55
3	E (7), X[b] (95)	7200	T (259)	55	L (1), D (6), E (1)	46	E (88)	27	E (15), V (4)	43
4	H (23)	122	E (357)	58	L (2), E (1), T (1)	44	P (78)	18	V (13)	55
5	I (40)	27	Y (353)	25	L (5)	39	V (72)	21	GAP[d]	68
6	A (21)	22	C[c]	43	I (7)	14			N (1)	35
7	E (10)	111	I (475)	36	P (3)	52			GAP[d]	38
8	R (7)	18	E (228)	36	Q (3)	37			GAP[d]	39[e]
9			T (142)	37	Q (5)	48				
10			G (168)	24						
11			A (196)	20						
12			P (125)	14						
13			E (103)	8						
14			G (72)	12						
15			S (18)	8						
16			F (48)	16						
17			L (37)	18						
18			V (30)	0						
19			R (17)	5						
20			X (0.8)[b]	94						
21			GAP[d]	49						
22			GAP[d]	37						
23			GAP[d]	41						
24			GAP[d]	29						
25			GAP[d]	38						

[a] Phenylthiohydantoin-amino acid (PTH-AA) residues identified from one-half of each sequencing reaction are given in one-letter code. The radioactivity recovered from the remaining half of the sequencing run is reported as counts per minute (cpm) minus blank. The data are representative of two sequencing reactions for each peptide peak. On the basis of the specific activities of the ^{3}H photoactive peptides, the following total amounts of cross-linked PLCγ1 peptides were applied to the sequencer: for peak b1, 660 pmol; for peak b3, 2450 pmol; for peak b4, 600 pmol; for peak c2, 670 pmol; and for peak c3, 460 pmol. [b] An X at a given cycle indicates that the PTH-AA did not migrate at a position for a known amino acid. The amount of derivative present was calculated from the radioactivity recovered from sequencing using the specific activity of the ^{3}H peptides. [c] Cys was detected (presumably as cystine) as a doublet migrating on the HPLC column immediately before and immediately after the PTH derivative of Tyr (Crankshaw & Grant, 1993). [d] PTH-AA residues whose identity was below the detection limit of the sequencer are indicated as GAP. [e] A significant amount of radioactivity (4230 cpm) remained on the sequencing filter after the sequencing reaction for peak c3 was completed.

when a single cross-linked peptide exhibits differential interactions with an HPLC column (Batra & Colman, 1986).

In order to identify peaks of radioactivity which may have arisen from remaining free photoaffinity label, a sample of peptide 992N-Bpa was irradiated under conditions similar to those of the cross-linking experiment. HPLC analysis under gradient conditions identical to those used to analyze cross-linking reactions showed that the major photolytic product of peptide 992N-Bpa eluted at 65.0 min (data not shown). Because of its similar elution time, peak b2 from the cross-linking reaction was not characterized further.

The remaining peaks, b1, b3, and b4, represented 11.5%, 21.3%, and 11.4% of the total radioactivity applied to the HPLC column, respectively. For both injections, radioactive peaks were concentrated *in vacuo* and analyzed by Edman peptide sequencing. The amounts of peptide analyzed in each sequencing run were as follows: for peak b1, 25% of the total (≈660 pmol); for peak b3, 50% (≈2620 pmol); and for peak b4, 25% (≈660 pmol) (amounts of peptide calculated from the specific activity of ^{3}H peptide 992N-Bpa). At each sequencing cycle, one-half of the phenylthiohydantoin-amino acid (PTH-AA) was identified by HPLC and the remainder analyzed by scintillation counting. The covalently modified residue was apparent for three reasons: the signal for a PTH-amino acid at a given cycle decreased dramatically, an unidentifiable PTH-AA was present, and some radioactive material was released from the sequencing filter (Miller, 1991). The sequences of the peptides obtained in this manner are given in Table 1, along with the positions at which radioactivity was released from the sequencing membrane. Peaks b1, b3, and b4 were each analyzed in two separate sequencing reactions (one for each of the two HPLC runs). Duplicate sequencing runs for these peaks gave identical peptide sequences.

Peak b1 corresponds to the sequence of the PLCγ1 N-terminal SH2 domain between residues Asp560 and Arg567, with a very low yield of the PTH-AA derivative of Glu and release of radioactivity at position 3 in the sequencing (see Table 1). These results indicate Arg562 as the site of cross-linking. On the basis of the specific activity of peptide 992N-Bpa, the amount of radioactivity released at cycle 3 corresponds to 95 pmol of labeled peptide. Peak b3 gave rise to the sequence between residues Leu568 and Arg586 of the SH2 domain, with release of a small amount of radioactivity (94 cpm), observation of an unknown amino acid phenylthiohydantoin at the next cycle, and sequence termination afterward, suggesting the site of attachment to be Glu587. Such abrupt sequence termination has been observed previously for sequencing of photo-cross-linked sites with benzophenone-type labels (Miller & Kaiser, 1988; Mourey *et al.*, 1993). We cannot rule out the possibility that the recovery of PTH-AA derivatives dropped below the detection limit, although the Edman sequencing reaction is routinely sensitive to low picomolar amounts of peptide. The sequenced peptide from peak b4 did not correspond to any sequence from the PLCγ1 N-terminal SH2 domain but was tentatively assigned as des-Asp^3 peptide 992N-Bpa by virtue

of 7/11 amino acid matches at the correct positions of peptide 992N-Bpa.

A similar strategy was employed to identify residues of the PLCγ1 SH2 domain which were modified by peptide 1068C-Bpa. In this case, SH2 domain and ^{3}H-acetylated peptide 1068C-Bpa were used in the photo-cross-linking reaction. Trypsin digestion, filtration, and HPLC analysis were carried out under conditions identical to those reported above for peptide 992N-Bpa. Again, >98% of the radioactivity was recovered in the Centricon-10 filtrate. Major peaks of radioactivity eluted from the HPLC column at 75.9 min (peak c1), 78.3 min (peak c2), and 83.6 min (peak c3) (Figure 2C). Peak c1 was not studied further because its retention time was similar to that of the major product of peptide 1068C-Bpa irradiation (75.4 min; data not shown). Peak c2 (representing 28.5% of the total radioactivity applied to the HPLC column) and peak c3 (20.4%) were concentrated and analyzed by peptide sequencing as described above. In this case, the amounts of peptide analyzed in each sequencing run were as follows: for peak c2, 25% of the total (≈670 pmol); for peak c3, 25% (≈460 pmol) (amounts of peptide calculated from the specific activity of ^{3}H peptide 1068C-Bpa). Peaks c2 and c3 were each analyzed in duplicate sequencing runs, and the sequences derived from these experiments were identical. The sequence of peak c2 corresponds to the sequence of the SH2 domain between residues Leu653 and Val657, with low yields of several phenylthiohydantoins at position 1 and concomitant release of radioactivity (Table 1). The amount of radioactivity released corresponds to 23 pmol of cross-linked peptide, based on the specific activity of peptide 1068C-Bpa. These results indicate Leu653 to be the modified residue. For peak c3, no radioactivity was released at any cycle of peptide sequencing, and several cycles gave rise either to multiple phenylthiohydantoins or to an absence of any phenylthiohydantoins. We were unable to characterize this peptide fragment further.

Mutation of the N-SH2 Region Inhibits Peptide Binding. To confirm the importance of residues within the conserved Phe-Leu-Val-Arg-Glu-Ser (FLVRES) motif of the SH2 domain for photo-cross-linking to peptide 992N-Bpa, quantitative cross-linking to wild-type and mutant PLCγ1 SH2 domains was carried out. The mutant proteins used were GST fusions consisting of an altered N-SH2 domain and an intact C-SH2. Two proteins, one with an Arg586 → Lys mutation in its N-SH2 and one with Ser588 → Cys, were constructed by site-directed mutagenesis. These mutations correspond to amino acid changes which greatly reduce the affinity of the Abl SH2 domain for the autophosphorylated epidermal growth factor receptor (Zhu *et al.*, 1993). Furthermore, both the NMR solution structure of the Abl SH2 domain (Overduin *et al.*, 1992) and the X-ray crystal structure of the Src SH2 domain (Waksman *et al.*, 1993) complexed to phosphotyrosine-containing peptides implicate the residues corresponding to Arg586 and Ser588 in binding to phosphotyrosine.

The stoichiometry of cross-linking was measured by a modified binding assay to glutathione−agarose. SH2 domains were irradiated for 5 min with ^{3}H peptide 992N-Bpa. The reaction mixtures were then incubated with glutathione−agarose, and the agarose beads were centrifuged, washed, and resuspended in Tris buffer. The tritium bound to glutathione−agarose was quantified by scintillation counting. Under these irradiation conditions the maximum amount of peptide 992N-Bpa cross-linked to the tandem SH2 domains of PLCγ1 was 0.52 mol of peptide/mol of SH2 domain (Table 2).

Table 2: Photo-Cross-Linking of Peptide 992-Bpa to Wild-Type and Mutant SH2 Domains

SH2	mol of peptide/ mol of protein[a]	incorporation (relative to wild type) (%)
WT	0.52 ± 0.02	100
Arg586 → Lys	0.26 ± 0.01	51
Ser588 → Cys	0.42 ± 0.01	80
Src	0.07 ± 0.005	12.5

[a] Reactions between [^{3}H]acetyl peptide 992N-Bpa and SH2 domains were carried out in triplicate as described in Materials and Methods. Incorporation of radioactivity into SH2 domains bound to glutathione−agarose was measured and converted into moles of peptide per mole of protein using the specific activity of peptide 992N-Bpa.

The results using mutant SH2 domains indicate that photoaffinity labeling of the mutants is significantly reduced relative to wild type (Table 2). Substitution of the Arg586 residue with lysine showed a 50% reduction in incorporation, and replacement of Ser588 with a cysteine resulted in a 20% decrease in incorporation. This is consistent with the sequencing results, in which peptide 992N-Bpa was covalently cross-linked at or near Glu587. Mutations at the neighboring amino acids either reduce the binding of peptide 992N-Bpa or, additionally or alternatively, result in a change in conformation such that bound peptide cross-links less effectively. Experiments carried out at 250 μM peptide 992N-Bpa indicated that the amount of cross-linking was roughly equivalent for wild-type and mutant SH2 domains (data not shown), suggesting that the defect is due to decreased binding. The cross-linking which does occur may be attributable to the presence of a wild-type C-SH2 domain in these fusion proteins. Finally, when a GST fusion protein containing the Src SH2 domain was irradiated together with tritiated peptide 992N-Bpa, only 12.5% of the peptide was incorporated (relative to PLCγ1). This reflects the absence of the (phospho)Tyr-Glu-Glu-Ile motif, required for recognition by the Src SH2 (Songyang *et al.*, 1993), from peptide 992N-Bpa (Table 2).

DISCUSSION

A primary advantage to using benzophenone-type photoaffinity labels is their high reactivity toward a variety of amino acid side chains (Williams & Coleman, 1982; Miller, 1991; Dormán & Prestwich, 1993). Irradiation of the benzophenone moiety of Bpa at 300−350 nm gives rise to a reactive triplet state which may insert into any carbon−hydrogen bond. Because bimolecular rate constants for reaction of triplet-state benzophenone with C−H bonds are 10^4−10^5 faster than with water (Helene, 1972), efficient labeling can be achieved in biological systems. In the present study, residues cross-linked in the PLCγ1 SH2 domain have been labeled by virtue of their spatial proximity to Bpa when EGFR peptides containing the photoactive amino acid are bound to the protein. As in any photoaffinity labeling study, it is possible that introduction of the Bpa label into the EGFR peptides results in a distortion of the complex structure. The finding that labeling reached a maximum of approximately 60% (Figure 1) could possibly be indicative of such a change; the following lines of evidence, however, argue against a drastic change in the conformation of the peptide: (1) the specificity of the interaction with the SH2 domain, and its

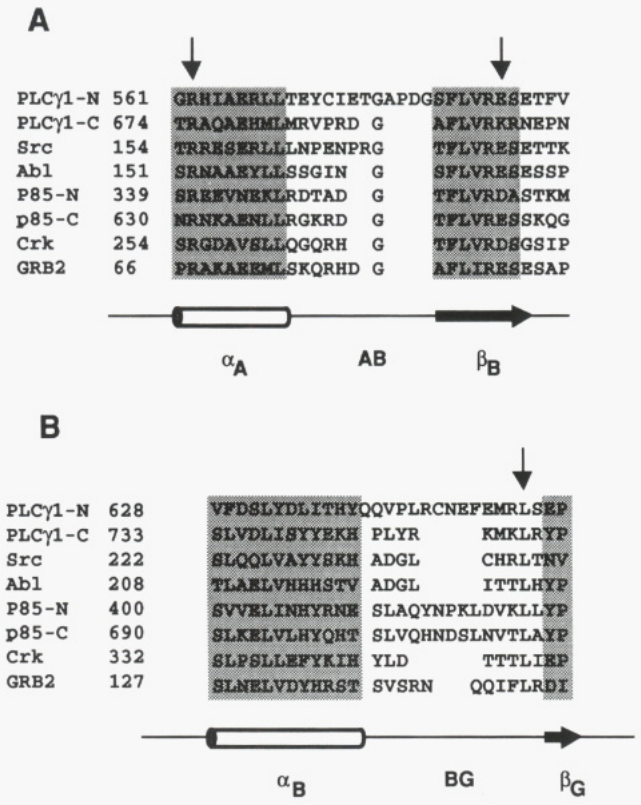

FIGURE 3: Alignment of SH2 sequences and positions of photocross-linking. The sequences of the SH2 domains from PLCγ1 (N- and C-terminal), Src, Abl, p85 subunit of PI 3′-kinase (N- and C-terminal), Crk, and Grb2 have been aligned on the basis of the alignment carried out by Waksman *et al.* (1993). Secondary structural elements from the Src SH2 domain are depicted schematically below the figure and are named according to the nomenclature proposed by Waksman *et al.* Positions of photoaffinity labeling are indicated by arrows. (A) Results of photoaffinity labeling by peptide 992N. (B) Results of photoaffinity labeling by peptide 1068C.

dependence on phosphorylated tyrosine, is preserved (Figure 1); and (2) the results obtained are in agreement with the topology determined from the Src SH2 domain crystal structure (Waksman *et al.*, 1993; see below). The lack of 100% cross-linking may be due to the GST−SH2 expression system used in the present study; it is possible that regions of PLCγ1 outside of the N-SH2 region are necessary for maximal interaction. Alternatively, regions of the EGFR not included in the phosphopeptide sequences may be important in the interaction. However, binding of PLCγ1 SH2 domains to EGFR was effectively blocked by low concentrations of non-Bpa phosphopeptides corresponding to the sequences of peptides 992N-Bpa and 1068C-Bpa (McNamara *et al.*, 1993).

Peptide 992N-Bpa cross-linked to two amino acids in the N-terminal portion of the N-SH2 region: Arg562 and a second residue, tentatively assigned as Glu587. The former is located in the αA helix and the latter in the βB sheet, near the highly conserved FLVRES motif [Figure 3A; nomenclature of Waksman *et al.* (1993) used throughout]. Although the crystal structure of the N-terminal SH2 domain of PLCγ1 has not been determined, the analogous residues in the Src SH2 structure are located close to the phosphotyrosine (Figure 4A). In particular, the corresponding arginine residue in the Src SH2 structure (Arg155) is involved in an amino−aromatic interaction with the ring of phosphotyrosine and donates hydrogen bonds to a phosphate

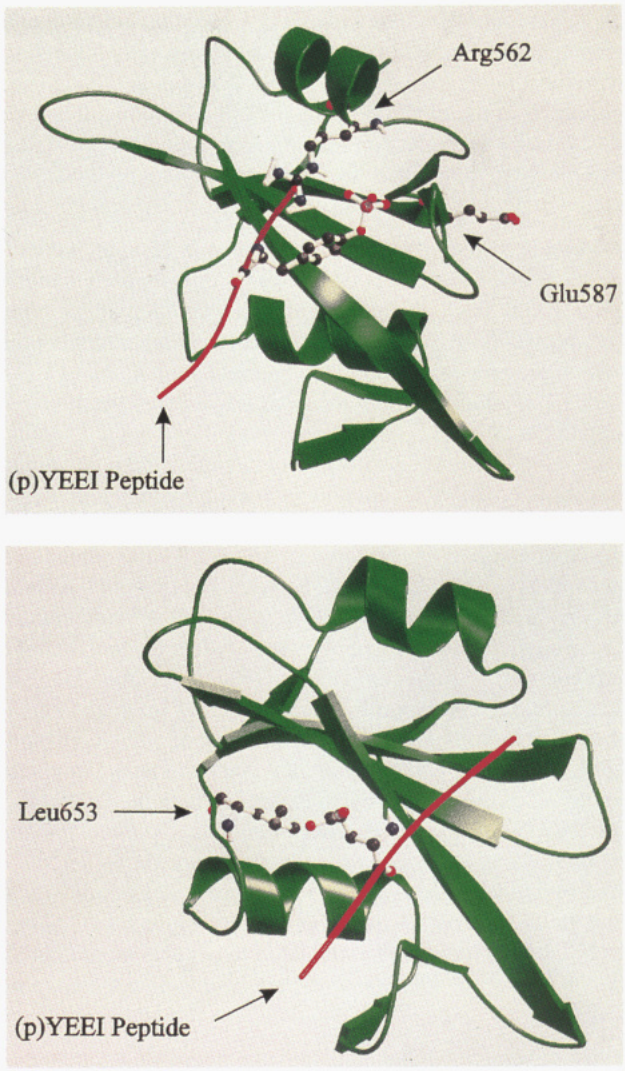

FIGURE 4: Positions of photoaffinity labeling displayed in the context of the Src SH2 domain structure. Ribbon diagrams of the crystal structure of the Src SH2 domain complexed to a high-affinity peptide were created using the program MOLSCRIPT. The SH2 domain is shown in green and the phosphotyrosyl peptide, which has the sequence Glu-Pro-Gln-(phospho)Tyr-Glu-Glu-Ile-Pro-Ile-Tyr-Leu, is shown in red. (A, top) Results of photoaffinity labeling by peptide 992N. The phosphotyrosine residue and the two amino acids modified by peptide 992C, Arg562 and Glu587 (PLCγ1 nomenclature), are shown in ball-and-stick format. (B, bottom) Results of photoaffinity labeling by peptide 1068C. The Glu +1 residue in the phosphopeptide and the site of cross-linking by peptide 1068C (Leu653 in the PLCγ1 nomenclature) are shown in ball-and-stick format.

oxygen (Figure 4A). These residues are most likely near the phosphotyrosine in the PLC−EGFR complex and mediate SH2 binding. Some radioactivity was also released in cycle 7 in peak b1, at the position corresponding to Glu566 in the N-SH2 sequence. This result may indicate that peak b1 contains a low level of cross-linked peptide 992N-Bpa at this site in addition to Arg562. Glu566 is on the same face of helix α_A as Arg562 but is located further from phosphotyrosine (Waksman *et al.*, 1993).

In the crystal structure of the Src SH2 domain, the analog to Glu587 (Glu176 in the Src numbering) does not appear to contact phosphotyrosine directly (Figure 4A). The residues adjacent to Glu587, however, make critical contacts with phosphotyrosine. Arg175 (corresponding to Arg586 of PLCγ1) is at the base of the phosphotyrosine-binding pocket and is engaged in an ion-pairing interaction with the

phosphate group. The hydroxyl group of Ser177 (corresponding to Ser588 of PLCγ1) also forms a hydrogen bond with phosphate oxygens. Amino acids near Glu587 are important in the function of the PLCγ1 SH2 domain as well, as shown by the reduction of peptide incorporation by mutant PLCγ1 SH2 domains. In some cases, the SH2 function seems to be destroyed by a single mutation (Arg586 → Lys).

Peptide 1068C-Bpa cross-links to one residue, Leu653, closer to the C-terminus of the domain in loop BG (Figure 3B). From the X-ray structural data, this loop is important for specificity in Src SH2−phosphotyrosine binding (Figure 4B; Waksman *et al.*, 1993). The BG loop in the PLCγ1 N-SH2 domain contains an insertion of five amino acids (relative to Src SH2), raising the possibility that the BG loops may adopt different conformations (Figure 3B). A similar insertion in the SH2 domain of the p85 subunit of the PI 3′-kinase results in a distinct conformation in which methionine residues are preferred at the +1 position relative to phosphotyrosine. The PLC N-SH2, like the p85 subunit, prefers hydrophobic residues at the position immediately C-terminal to the phosphotyrosine, whereas the Src SH2 prefers Glu (Songyang *et al.*, 1993). In the Src SH2 structure, the corresponding leucine (Leu242) packs against the side chain of a tryptophan residue near the N-terminus of the domain (Waksman *et al.*, 1993). The hydrophobic nature of many of the nearby side chains in the BG loop of the N-SH2 (Val642, Pro643, Leu644, Cys646, Phe649, Met651) may contribute to a binding pocket to select for hydrophobic residues at the +1 position. Residues Pro643 and Leu644 occur at the BG3 and BG4 positions, respectively, which have been implicated in peptide recognition at the +3 position (Songyang *et al.*, 1993; Waksman *et al.*, 1993).

In summary, the results of the photoaffinity labeling experiments suggest that the overall topology of the PLCγ1 N-SH2 is similar to that of the Src SH2 domain. The strength of the photoaffinity labeling approach used here is that it allows sequencing and identification of individual amino acids involved in interaction with EGFR autophosphorylation site peptides. Residues which were photo-cross-linked to peptide 992N-Bpa are likely to be involved in recognition of phosphotyrosine; these data are corroborated by the results of mutagenesis experiments reported here (Table 2) and elsewhere for other SH2 domains [e.g., Zhu *et al.* (1993)]. The results of photoaffinity labeling studies with peptide 1068C-Bpa indicate that specificity determinants for residues at the +1 position of bound phosphoproteins reside (at least in part) in the BG loop of the PLCγ1 N-SH2. As reported previously for the phosphatidylinositol 3′-kinase p85 SH2 domain (Williams & Shoelson, 1993), peptide-based photoaffinity labeling provides a powerful approach for analyzing interactions which occur at SH2−phosphoprotein interfaces.

ACKNOWLEDGMENT

The authors thank James L. Hicks (Parke-Davis) for acetylation of the radiolabeled peptides and Prof. John Kuriyan (Rockefeller University) for suggestions during the course of this study. Protein sequencing was performed by the Center for Analysis and Synthesis of Macromolecules at Stony Brook, supported by NIH Grant RR02427, and the Center for Biotechnology.

REFERENCES

Anderson, D., Koch, C. A., Grey, L., Ellis, C., Moran, M. F., & Pawson, T. (1990) *Science 250,* 979−982.

Batra, S. P., & Colman, R. F. (1986) *Biochemistry 25,* 3508−3515.

Brunner, J. (1993) *Annu. Rev. Biochem. 62,* 483−514.

Crankshaw, M. A., & Grant, G. A. (1993) Identification of Modified PTH-Amino Acids in Protein Sequence Analysis, *Bulletin of the Association of Biomolecular Research Facilities,* 1st ed., Association of Biomolecular Research Facilities, Bethesda, MD.

Dormán, G., & Prestwich, G. D. (1993) *Chemtracts: Org. Chem. 6,* 131−138.

Fantl, W. J., Johnson, D. E., & Williams, L. T. (1993) *Annu. Rev. Biochem. 62,* 453−481.

Helene, C. (1972) *Photochem. Photobiol. 16,* 519−522.

Kauer, J. C., Erickson-Viitanen, S., Wolfe, H. R., Jr., & DeGrado, W. F. (1986) *J. Biol. Chem. 261,* 10695−10700.

Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24,* 946−950.

Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D., & Schlessinger, J. (1992) *Cell 70,* 431−442.

Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T., & Sclessinger, J. (1990) *EMBO J. 9,* 4375−4380.

Mayer, B. J., & Baltimore, D. (1993) *Trends Cell Biol. 3,* 8−13.

Mayer, B. J., Hamaguchi, M., & Hanafusa, H. (1988) *Nature 332,* 272−275.

McNamara, D. J., Dobrusin, E. M., Zhu, G., Decker, S. J., & Salteil, A. R. (1993) *Int. J. Pept. Protein Res. 42,* 240−248.

Miller, W. T. (1991) *Methods Enzymol. 200,* 500−508.

Miller, W. T., & Kaiser, E. T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 5429−5433.

Mourey, R. J., Estevez, V. A., Maracek, J. F., Barrow, R. K., Prestwich, G. D., & Snyder, S. H. (1993) *Biochemistry 32,* 1719−1726.

Overduin, M., Rios, C. B., Mayer, B. J., Baltimore, D., & Cowburn, D. (1992) *Cell 70,* 697−704.

Pawson, T., Olivier, P., Rozakis-Adcock, M., McGlade, J., & Henkemeyer, M. (1993) *Philos. Trans. R. Soc. London, B 340,* 279−285.

Rhee, S. G., & Choi, K. D. (1993) *Adv. Second Messenger Phosphoprotein Res. 28,* 35−61.

Rotin, D., Margolis, B., Mohammadi, M., Daly, R. J., Daum, G., Li, N., Fischer, E. H., Burgess, W. H., Ullrich, A., & Schlessinger, J. (1992) *EMBO J. 11,* 559−567.

Smith, D. B., & Johnson, K. S. (1988) *Gene 67,* 31−40.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., & Cantley, L. C. (1993) *Cell 72,* 767−778.

Stewart, J. M., & Young, J. D. (1984) *Solid Phase Peptide Synthesis,* 2nd ed., Pierce Chemical Co., Rockford, IL.

Ullrich, A., & Schlessinger, J. (1990) *Cell 61,* 203−212.

Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D., & Kuriyan, J. (1993) *Cell 72,* 779−790.

Williams, K. P., & Shoelson, S. E. (1993) *J. Biol. Chem. 268,* 5361−5364.

Williams, N., & Coleman, P. S. (1982) *J. Biol. Chem. 257,* 2834−2841.

Zhu, G., Decker, S. J., & Saltiel, A. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 9559−9563.

Zhu, G., Decker, S. J., Mayer, B. J., & Saltiel, A. R. (1993) *J. Biol. Chem. 268,* 1775−1779.